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Tumor Recognition following Vγ9Vδ2 T Cell Receptor Interactions with a Surface F1-ATPase-Related Structure and Apolipoprotein A-I

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Summary

Vγ9Vδ2 T lymphocytes, a major γδ T lymphocyte subset in humans, display cytolytic activity against various tumor cells upon recognition of yet uncharacterized structures. Here, we show that an entity related to the mitochondrial F1-ATPase is expressed on tumor cell surface and promotes tumor recognition by Vγ9Vδ2 T cells. When immobilized, purified F1-ATPase induces selective activation of this lymphocyte subset. The Vγ9Vδ2 T cell receptors (TCR) and the F1-ATPase also bind a delipidated form of apolipoprotein A-I (apo A-I), as demonstrated by surface plasmon resonance. Moreover, the presence of apo A-I in the culture medium is required for optimal activation of Vγ9Vδ2 T cells by tumors expressing F1-ATPase. This study thus describes an unanticipated tumor recognition mechanism by Vγ9Vδ2 lymphocytes and a possible link between γδ T cell immunity and lipid metabolism.

Introduction

Through their αβ TCR, peripheral T lymphocytes classically recognize foreign peptidic antigens bound to class I or class II major histocompatibility complex (MHC) molecules. Besides these "conventional" T cells, other subsets expressing either αβ or γδ TCR react against a more heterogeneous set of nonpeptidic compounds, either in a native form or in association with conserved MHC-related molecules (Beckman et al., 1994; Moody et al., 1997; Spada et al., 2000).

In humans, the vast majority of peripheral blood γδ T cells use a particular combination of variable regions (Vγ9 and Vδ2) to form their TCR. These Vγ9Vδ2 T cells are activated in a TCR-dependent fashion by several small phosphorylated (Constant et al., 1994; Tanaka et al., 1994) or aminated (Bukowski et al., 1999) alkyl molecules. Vγ9Vδ2 T cell activation by these compounds requires intercellular contact, thus suggesting some kind of antigen presentation (Lang et al., 1995; Morita et al., 1995). Vγ9Vδ2 cells also react against several fresh or cultured tumors in vitro and exhibit both cytolytic activity and production of inflammatory cytokines (TNFα and IFNγ). This activity is tightly regulated by NK-like receptors for MHC class Ia and class Ib antigens that are frequently expressed by this T cell subset (Fisch et al., 1997; Halary et al., 1997). Thus, besides their role in immunity against viral and bacterial infections (Bukowski et al., 1994; Ramsburg et al., 2003), γδ T cells are probably involved in tumor surveillance (Bukowski et al., 1995; Fisch et al., 1997; Wu et al., 2002). The involvement of γδ T cells in tumor immunity is further supported by in vivo experiments showing inhibition of Burkitt's lymphoma growth upon human Vγ9Vδ2 T cell transfer into severe combined immunodeficient mice (Malkovska et al., 1994), and the implication of murine γδ T cells in early control of chemically induced cutaneous malignancies (Girardi et al., 2001, 2003).

Some human γδ T cells of the Vδ1 subset react toward the nonclassical MHC molecules MICA and MICB, which are stress induced. It is not clear, however, if this recognition is mediated by TCR (Groh et al., 1998) or by NKG2D molecules (Wu et al., 2002). In the case of Vγ9Vδ2 T cells, the tumor antigens targeted by the TCR remain unknown so far. Recognition of tumor cells, such as the Daudi lymphoma by Vγ9Vδ2 T cells, correlates with production of mevalonate metabolites such as isopentenyl pyrophosphate (IPP) (Gober et al., 2003). This phenomenon could account for the dual recognition of soluble nonpeptidic and tumor antigens by the same T cell population. However, efficient activation of Vγ9Vδ2 T cells requires millimolar concentrations of IPP, a concentration range that is unlikely to be reached in the extracellular medium in physiological conditions. Thus, tumor cell recognition probably also involves stable cell surface components. In this work, we show that a surface structure related to the mitochondrial F1-ATPase (F1) is present on the surface of Vγ9Vδ2-susceptible tumors, selectively binds to the Vγ9Vδ2 TCR, and is implicated in tumor recognition by Vγ9Vδ2 cells. This unexpected mechanism of tumor recognition is also modulated by ligands for F1 such as apo A-I.

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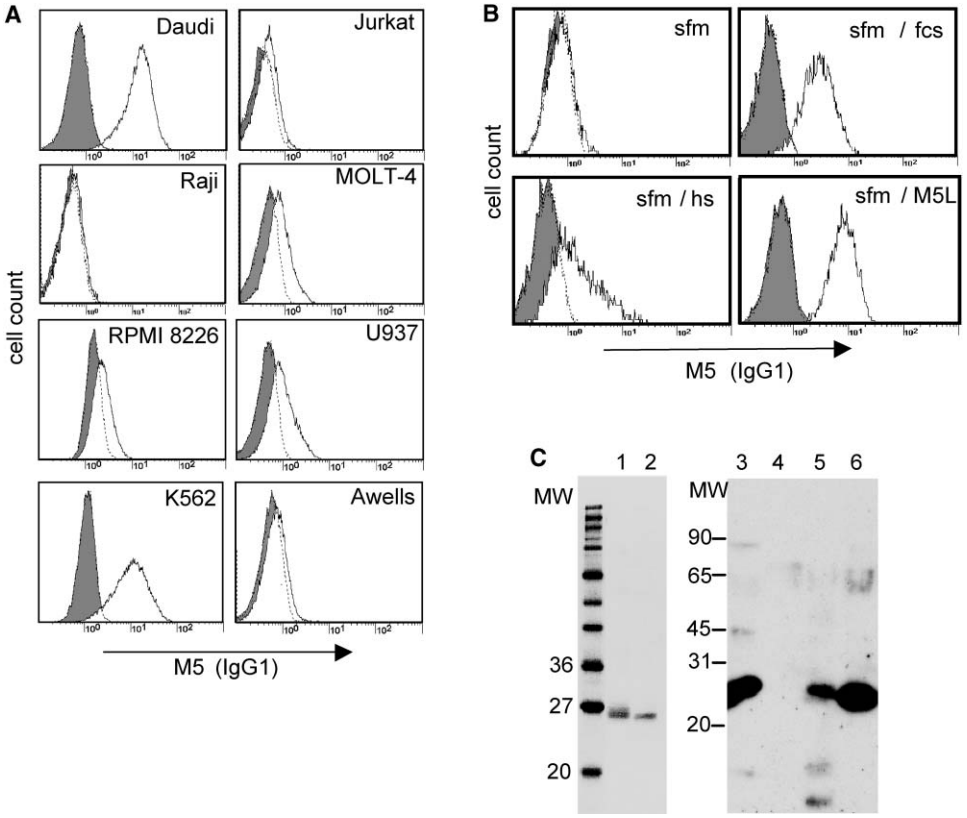

Figure 1. Extracellular Apo A-I Binds to Tumor Cells

(A) Indirect immunofluorescence staining with M5 mAb or a control IgG1 antibody (shaded histograms) of human cell lines cultured in presence of fetal calf serum (FCS). Daudi and Raji are Burkitt's lymphomas cell lines; Awells is a B-lymphoblastoid cell line; K562 is an erythroid leukemia cell line; RPMI 8226 is a B-cell myeloma cell line; and U937 is a monocytic leukemia cell line. MOLT-4 and Jurkat are T cell leukemia cell lines.
(B) Daudi cells were depleted of serum components by overnight culture in SFM and subsequently incubated in PBS supplemented with either 10% FCS (SFM/FCS), 10% human serum (SFM/HS), or 1% BSA plus 100 μg/ml of M5L (SFM/M5L) before staining as in (A).
(C) Left, Coomassie blue staining of M5-immunopurified material from FCS (lane 1) and HS (lane 2) after nonreducing SDS-polyacrylamide gel electrophoresis (PAGE). Right, immunoblot after reducing PAGE with antihuman apo A-I antibody 4H1 on HS (lane 3), FCS (lane 4), HDL (lane 5), and hM5L (human serum ligand of M5 mAb, lane 6).

Results

Modulation of Vγ9Vδ2-Mediated Tumor Cell Recognition by Apo A-I and Apo A-I-Specific mAb

In an attempt to characterize surface tumor antigens involved in the activation of Vγ9Vδ2 T cells, we raised murine monoclonal antibodies (mAb) against the Burkitt's lymphoma Daudi, a cell line that is lysed by a large majority of Vγ9Vδ2 T cell clones (Davodeau et al., 1993; De Libero et al., 1991). mAb were selected for their ability to differentially bind to Daudi and Raji (a nonactivating Burkitt's lymphoma) and to inhibit recognition of Daudi cells by Vγ9Vδ2 T cells. One mAb (#M5A12D10, hereafter referred to as M5) that fulfilled both criteria was selected for further studies. M5 mAb stained several cell lines killed in vitro by Vγ9Vδ2 T cells, such as Daudi and K562, but did not stain several Vγ9Vδ2-resistant tumors, such as Raji lymphoma and Awells B lymphoblastoid cells (Figure 1A). As M5 mAb binding to tumor cells was drastically decreased when cells were cultured in serum-free medium (SFM) (Figure 1B), this mAb was subsequently used to immunopurify a putative ligand from bovine serum. A protein (M5L) of apparent molecular weight of ~26 kDa was immunoprecipitated by M5 mAb, and mass spectrometry analysis of tryptic peptide digests identified apo A-I as the likely ligand. M5 mAb immunopurified a similar protein from human serum (hM5L), and its identity to apo A-I was confirmed by its reactivity in immunoblotting experiments with the antihuman apo A-I monoclonal antibody 4H1 (Figure 1C). Accordingly, like M5 mAb, 4H1 stained Daudi, but not Raji cells, after incubation with a human lipid-free form of human apo A-I prepared from high-density lipoprotein particles (HDL-apo A-I) by ion exchange chromatography (data not shown).

M5 decreased in vitro lysis of Daudi cells mediated by Vγ9Vδ2 T cell clones or a polyclonal Vγ9Vδ2 T cell line. By contrast, Daudi cell lysis by a reactive Vγ8Vδ3 T cell clone (#73R9) was not affected by M5 mAb (Figures 2A and 2B). In order to assess the putative involvement of apo A-I in Vγ9Vδ2 cytolytic activity, cytotoxicity assays were performed in SFM by using target cells depleted of serum after an overnight culture in SFM (Figure 2B). The cytolytic activity of Vγ9Vδ2 T cell clones against Daudi cells was decreased in serum-free conditions and restored by addition of serum during the cyto-

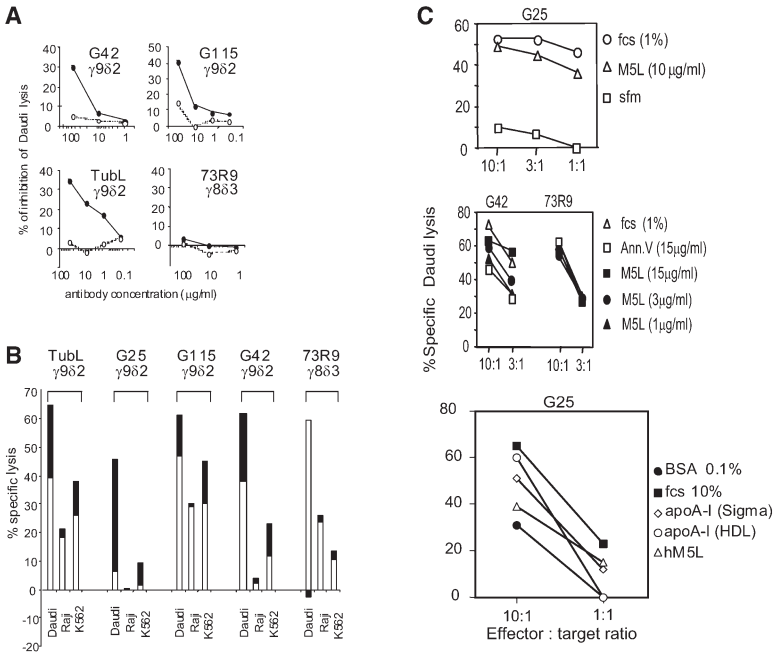

Figure 2. Apo A-I Modulates Tumor Lysis

TubL is a polyclonal Vγ9Vδ2 line; G25, G115, G42, and 73R9 are T cell clones expressing the indicated TCR.

(A) Standard ^{51}Cr-release assay on Daudi cells with, as effectors, T cell clones and lines bearing the indicated TCR variable regions. The assay was performed in medium containing 10% FCS (effector:target ratio, 10:1) and variable concentrations of M5 mAb (closed circles) or of a control IgG1 mAb (open circles). Specific lysis of Daudi cells for G42, G115, TubL, and 73R9 was 56.7%, 61.3%, 52.9%, and 63.5% respectively.

(B) Effector T cells (top) and target cell lines (horizontal axis) were serum depleted and cocultured for 2 hr in SFM (white bars) at an effector:target ratio of 10:1. Black bars indicate the increase of lysis efficiency in wells supplemented with 3% FCS.

(C) ^{51}Cr-release assays were performed in SFM to assess dependence on serum and apo A-I of Daudi cytolysis by different effector T cell populations. M5L (bovine serum component immunopurified with M5 antibody), and apo A-I preparations were added at the 100 μg/ml or at the indicated concentration to assess their ability to substitute for serum (FCS). Recombinant Annexin V was used as a control purified protein.

toxicity assay. Similarly, addition of purified M5L increased Vγ9Vδ2 T cell-mediated lysis in a dose-dependent manner. By contrast, Daudi cell lysis by the control Vγ8Vδ3 T cell clone was neither affected by serum deprivation nor by addition of M5L (Figures 2A and 2B). A similar effect on Vγ9Vδ2 T cell cytotoxicity was observed by using HDL-apo A-I or a commercial apo A-I preparation, thus ruling out a nonspecific stimulatory effect (Figure 2C).

The effect of apo A-I on Daudi cell lysis significantly varied from one Vγ9Vδ2 T cell clone to another, with some clones, like #G25, being highly dependent on apo A-I, whereas other ones, like #G115, were less affected. This clonal variability may involve TCR-dependent and TCR-independent mechanisms. Irrespective of this issue, the fact that cytotoxicity of Vγ9Vδ2 clones, unlike that of other γδ or αβ clones, was in most instances sensitive to apo A-I suggested specific contribution of apo A-I in Vγ9Vδ2-mediated recognition of tumor cells.

Implication of F1, an Apo A-I Receptor, in Tumor Cell Recognition by Vγ9Vδ2 T Cells

Although apo A-I modulated tumor cell recognition by Vγ9Vδ2 cells, it could not be the only factor controlling tumor susceptibility to Vγ9Vδ2 cell recognition owing to its ubiquitous and soluble nature. We therefore undertook a detailed analysis of apo A-I receptors on several tumor cell lines. Scatchard analysis of the binding of iodinated apo A-I to Daudi cells indicated the presence of a single binding receptor on these cells ($K_D = 0.8 \times 10^{-7}$ M) (Supplemental Figure S1 available online at http://www.immunity/com/cgi/content/full/22/1/71/DC1). Because Daudi cells do not express the long-known apo A-I receptors Scavenger Receptor B1 (Murao et al., 1997) or ABCA-1 (Chambenoit et al., 2001) (data not shown), we studied expression of a high-affinity apo A-I receptor recently identified on hepatocytes, namely the ATP synthase/F1-ATPase complex (Martinez et al., 2003). Subunits of this complex were clearly detected on several tumor cell lines (Figure 3). In particular, Daudi, K562, and RPMI 8226 were stained by mAb against the α chain of F1-ATPase (αF1), whereas Raji, leukemic T cells, and B-LCL were not (Figure 3A). Four kidney tumors that were lysed by Vγ9Vδ2 CTL (E.S. and M.B., unpublished data) also expressed an αF1-related surface component (Figure 3C). The β subunit of F1 (βF1) was detected on Daudi, K562, and U937, a monocytic line not consistently killed by Vγ9Vδ2 T cells. This chain was undetectable on RPMI 8226 and kidney tumors (Figures 3B and 3C). Therefore, tumor susceptibility to Vγ9Vδ2 lysis strongly correlates with expression of the αF1 subunit. Moreover, M5 staining correlates with the detection of βF1, in agreement with binding of apo A-I to the βF1 subunit on hepatocytes (Martinez et al., 2003).

To further evaluate the possible involvement of F1 in tumor cell recognition by Vγ9Vδ2 T cells, we investigated the effect of F1-specific mAb on lymphokine production induced upon short-term incubation of Vγ9Vδ2 T cell clones with Daudi cells. IFNγ secretion was strongly inhibited by anti-αF1, anti-βF1, and M5 antibodies when compared to control mAb. This inhibition was not due to a nonspecific toxic effect, as indicated by the lack of inhibition of IPP-induced Vγ9Vδ2 T cell activation by M5 or anti-βF1 mAb (Figure 4).

To confirm the implication of F1 in Vγ9Vδ2 T cell activation, latex beads were coated with purified bovine mitochondrial F1 complex and/or apo A-I and tested for their ability to stimulate Vγ9Vδ2 and control T cell populations (Figure 5). Coculture with beads coated with bovine F1 induced strong TNFα secretion by Vγ9Vδ2

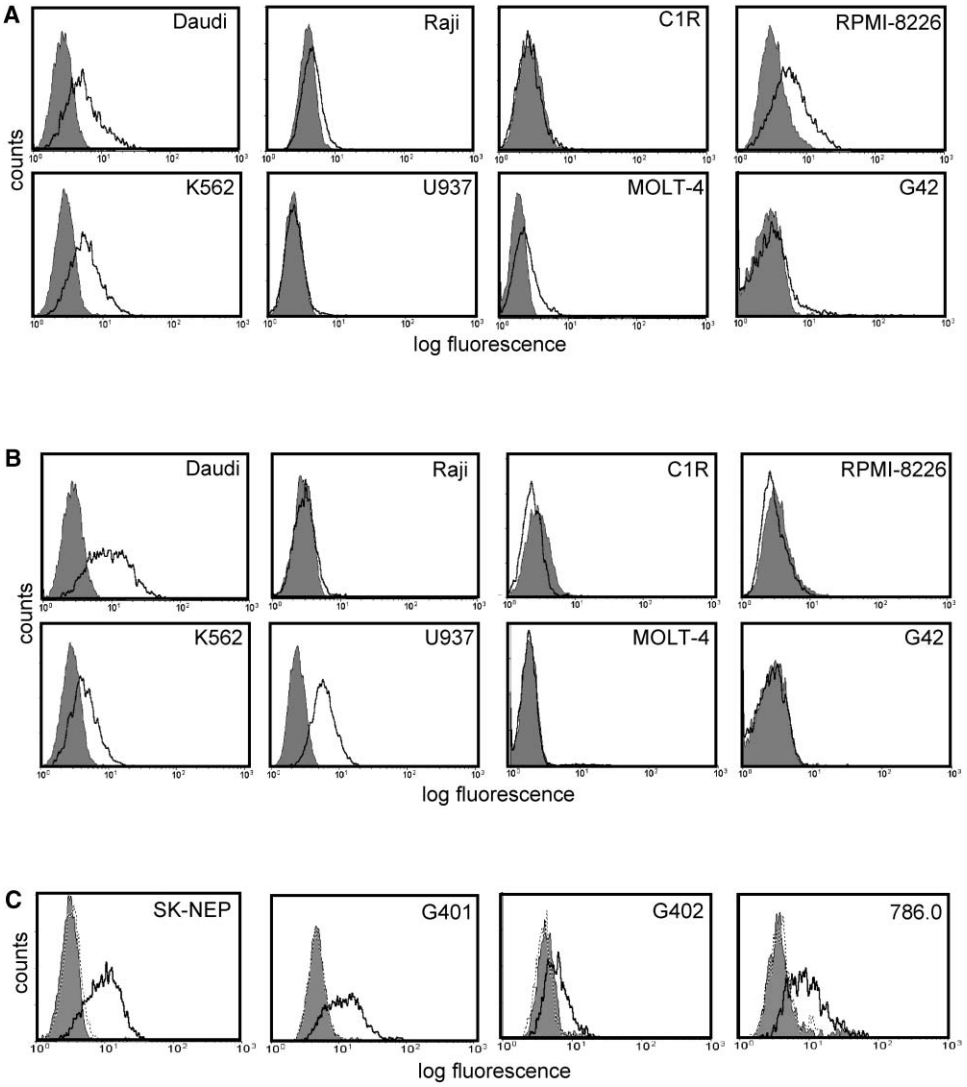

Figure 3. Expression of F1-Related Structures on Tumor Cells

(A and B) Indirect immunofluorescence surface staining of hematopoietic tumor lines with (A) anti-αF1 and (B) anti-βF1 mAb.
(C) Four kidney tumors sensitive to Vγ9Vδ2 lysis were tested for expression of F1 by FACS staining by using control IgG (shaded histogram), anti-αF1 (dark line), and anti-βF1 mAb (dotted line).

clones, whereas Vγ8Vδ3 and αβ clones, otherwise able to produce TNFα after stimulation by phorbol myristate acetate and calcium ionophore, were not activated (Figure 5A). Immobilized apo A-I alone failed to stimulate Vγ9Vδ2 T cell clones. Similar experiments performed in serum-free conditions showed a stimulatory activity of beads carrying F1 alone and a relatively minor increase of this stimulatory activity after addition of soluble apo A-I (Figure 5B). This would suggest an accessory role played by apo A-I in F1-induced Vγ9Vδ2 T cell activation. However, carryover of apo A-I or related apolipoproteins by effector cells could not be formally ruled out in such experiments. Consistent with a highly restricted stimulatory activity of F1 on the Vγ9Vδ2 T cell subset, beads carrying F1 and apo A-I induced selective TNFα production by a large fraction of $V\delta2^+$ T cells when added onto fresh peripheral blood lymphocytes (PBL) but had no significant effect on αβ and NK cell populations (Figure 5C).

Cognate Interactions between Vγ9Vδ2 TCR, F1, and Apo A-I

To further document the implication of the TCR in F1- and apo A-I-mediated activation of Vγ9Vδ2 T cells, we studied interactions between purified F1, apolipoproteins, and soluble recombinant forms of γδ TCRs in acellular systems. When tetramerized by fluorescent streptavidin, the Vγ9Vδ2 TCR derived from the G115 clone (Allison et al., 2001) stained Daudi, K562, and more weakly RPMI 8226, but neither Raji nor B lymphoblastoid cell lines. Vγ8Vδ3 TCR derived from clone 73R9 stained Daudi and, to a much lesser extent, K562 cells (Supplemental Figure S1 and data not shown). This strongly suggests that the two different TCR target different ligands on Daudi cells, in agreement with the distinct lysis patterns of the original T cell clones (see Figure 2). Interaction of monomeric soluble TCR with apo A-I and F1 was then tested by surface plasmon resonance (SPR) analysis (Figure 6). In these conditions, soluble G115

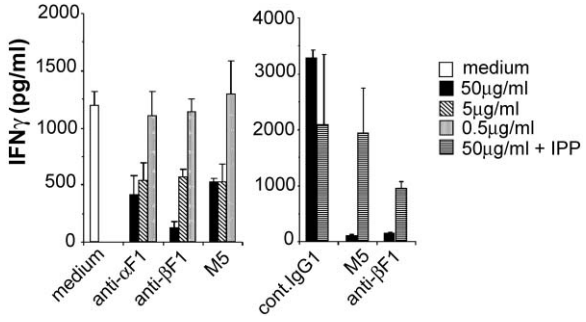

Figure 4. Modulation of Lymphokine Secretion by Anti-F1 Antibodies

Left, Daudi cells were incubated in serum, washed, and incubated with serum-depleted Vγ9Vδ2 cells (clone G42) in SFM, in the presence of the indicated concentration of antibodies, and the production of γ-interferon was measured after a 20 hr coculture. Right, to exclude a possible toxicity of antibodies on responding cells, antibodies were dialyzed, and isopentenyl pyrophosphate (IPP, 2 μg/ml) was added in some cultures.

TCR, unlike 73R9 TCR, specifically bound to apo A-I immobilized onto SPR chips (K_D = 0.8 μM). Vγ9Vδ2 also bound to SPR chips coated with soluble bovine F1 complex (K_D = 1.5 μM), unlike control Vγ8Vδ3 TCR. These results demonstrated the occurrence of specific interactions between the Vγ9Vδ2 TCR, apo A-I, and F1, possibly through formation of a trimolecular complex. In order to analyze further the respective contribution of apo A-I and F1, another experimental setting was used. Purified ATP synthase was immobilized onto plastic wells and binding of soluble biotinylated recombinant TCR was quantitated by a streptavidin-alkaline phosphatase colorimetric assay. Soluble Vγ9Vδ2 TCR bound to ATP synthase-coated wells and addition of hM5L onto immobilized ATP synthase increased subsequent TCR binding by 30%–40% under these conditions (Figures 7A and 7B). This binding was competed by anti-clonotypic mAb, but not by irrelevant Vγ- or Vδ-specific mAb (Figure 7C). TCR binding was inhibited in a specific and dose-dependent manner by anti-αF1 or anti-βF1 antibodies (Figures 7A and 7B). Finally, hM5L increased TCR binding even in the presence of anti-F1 antibodies, suggesting that these antibodies do not impede the bridging of TCR and F1 by hM5L. Moreover, the anti-apo A-I antibody 4H1 did not prevent TCR binding to F1 but inhibited the binding increment provided by hM5L (Figure 7B). These results suggest that apo A-I acts as a stabilizer of the TCR-F1 interaction.

Discussion

In this work, we have identified two ligands of the Vγ9Vδ2 TCR on tumor targets. The first one is immunologically related to the ATP synthase/F1-ATPase normally expressed on the internal membrane of mitochondria and is detected with anti-αF1 and/or anti-βF1 antibodies. The second one corresponds to apo A-I, a recently described ligand for F1. The binding parameters of the interactions between Vγ9Vδ2 TCR and both ligands are very similar to those reported for several other nonconventional TCR such as the murine γδ T cell clone G8 (Crowley et al., 2000) and several CD1d-restricted NKT cell clones (Brossay et al., 1998; Stanic et al., 2003). In particular, binding affinity of Vγ9Vδ2 TCR for F1 and apo A-I are in the 10^{-6}–10^{-7} M range and thus can be considered as high-affinity interactions when compared to the binding affinities of αβ TCR for peptide/MHC complexes (classically in the 10^{-4}–10^{-6} M range [Davis et al., 1998]). As for murine γδ T cells, high binding affinity of Vγ9Vδ2 TCR for its presently described ligands is mainly accounted for by a very fast association rate and a rather slow dissociation rate. As previously suggested (Crowley et al., 2000), these features may allow Vγ9Vδ2 TCR to efficiently catch transient/unstable complexes formed between F1, apoA-I, and possibly other tumor ligands (such as phosphoantigens, see below). Besides, these results are consistent with the immunoglobulin-like features of Vγ9Vδ2 TCR suggested by a recent crystallographic study (Allison et al., 2001).

Although unexpected, the ectopic detection of F1 on the cell surface is not unprecedented as it has already been described on K562 cells (Das et al., 1994), endothelial cells (Moser et al., 1999), and hepatocytes (Martinez et al., 2003). Furthermore, the presence of F1 within lipid rafts on HeLa cells has been recently reported (Bae et al., 2004). Different functions have been attributed to this structure, including an immunoregulatory role in the case of tumors (Das et al., 1994), angiostatin receptor (Moser et al., 1999), and regulation of lipoprotein transport through high-affinity apo A-I binding (Martinez et al., 2003). The structure found on Daudi and K562 cells is not a crossreactive entity unrelated to F1 because bovine F1 complex, when immobilized on plastic or latex beads, also binds soluble Vγ9Vδ2 TCR and activates specifically Vγ9Vδ2 cells.

Available monoclonal antibodies reveal a striking dissociation between the expression of the αF1 and βF1 subunits on tumor cell lines, suggesting some form of polymorphism of this structure. Although the mitochondrial receptor has similar stimulatory properties for γδ cells, the structure displayed on the plasma membrane may use some of its components to perform different functions. The isolated expression of αF1 or βF1 subunits on the cell surface seems unlikely, as these subunits do not contain any membrane anchor domains. Thus, we would favor the possibility that on some cells the βF1 or the αF1 chains are either hindered or replaced by yet unknown structures. A predominant role of αF1 as a Vγ9Vδ2 TCR target is suggested by the correlation between tumor sensitivity to Vγ9Vδ2 cytotoxicity and surface expression of αF1. Nevertheless, its implication in a protein complex is supported by the inhibitory effect of anti-βF1 antibodies on TCR binding and cytokine production. Whether these components are also involved in an enzymatic complex on tumor cells is not known yet.

Antibodies to apo A-I moderately affect target lysis by Vγ9Vδ2 effectors. Similarly, coimmobilization of apo A-I with F1 has a moderate effect on T cell stimulation and TCR binding. Immobilization of apo A-I by itself is not sufficient to promote stable binding of biotinylated TCR (data not shown) or to activate Vγ9Vδ2 clones (Figure 5). This suggests a secondary role of apo A-I. Nonetheless, addition of serum is required for optimal cytolytic activity of Vγ9Vδ2 T lymphocytes, and this effect

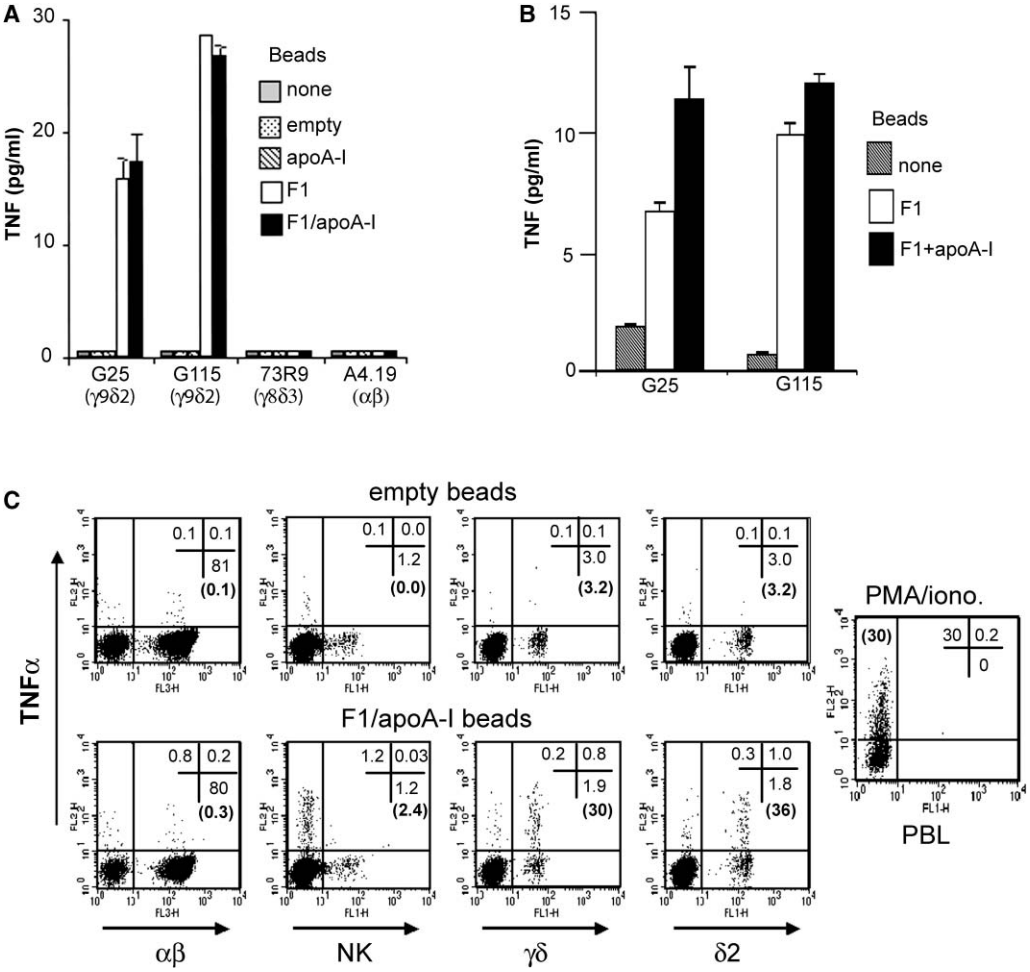

Figure 5. Induction of Vγ9Vδ2 T Cell Lymphokine Secretion by Immobilized F1

HDL-derived apo A-I and F1 were immobilized on latex beads, and these were used to stimulate T cell populations. Legend: none, no stimulation; empty, beads saturated with BSA; apo A-I, beads coated with apo A-I only; F1, beads coated with the F1 extra-membrane subunit of bovine F1; and F1/apo A-I, beads coated with both protein preparations.

(A) T cell clones were activated with protein-coated beads in medium supplemented with human serum, and TNFα secretion was measured in the culture supernatant after 4 hr. The capacity of γδ clones G25, G115, and 73R9 to secrete significant amounts of TNFα upon stimulation was checked by using Daudi cells as target cells as well as PMA/ionomycin or PHA. The αβ clone A4.19 secretes saturating quantities of TNFα upon PMA/ionomycin or PHA stimulation (not shown).

(B) Stimulation was performed in the absence of exogenous serum (SFM), and purified HDL-derived apo A-I was added in some cultures (black bars).

(C) Fresh PBL were activated with indicated beads for 4 hr in the presence Brefeldin A. Cells were then stained with antibodies to lymphocyte subsets, fixed, and permeabilized, and TNFα accumulation was analyzed after intracellular staining by flow cytometry. PMA/ionomycin stimulation was used as a control for TNFα-producing cells in the total population. Percentages indicating TNFα-producing cells within analysis quadrants and within each particular subset (between brackets) are shown.

is mimicked by exogenous apo A-I. Unlike ELISA experiments, SPR analysis allows detection of transient interactions and reveals a possible binding site between G115 TCR and apo A-I. This suggests that apo A-I somehow stabilizes/increases TCR-F1 interaction by bridging distinct binding sites on the TCR and the βF1 chain. Such an assumption is supported by the increased binding of biotinylated soluble TCR to immobilized F1 complexes containing apo A-I (Figure 7). The effect of serum deprivation on Daudi lysis by Vγ9Vδ2 T cells greatly varies from one clone to another, with G115 clones being quite resistant to this deprivation. Thus, the G115-TCR may not be the best receptor to use in order to assess the contribution of apo A-I to TCR binding. Nevertheless, apo A-I could perform a chaperone-like function in these interactions. In this regard, it will be interesting to determine whether other chaperones such as Hsp60 (Fisch et al., 1990), which has been previously involved in Vγ9Vδ2 T cell-mediated recognition of Daudi cells and is known to bind to scavenger receptors possibly related to apo A-I receptors, could perform a similar role.

In a recent work, Gober et al. (2003) found an increased accumulation of ubiquitous endogenous IPP in tumors activating Vγ9Vδ2 T cell clones. Such accumulation could account for continuous production of these antigens, so that cells remain activatory after washing. In

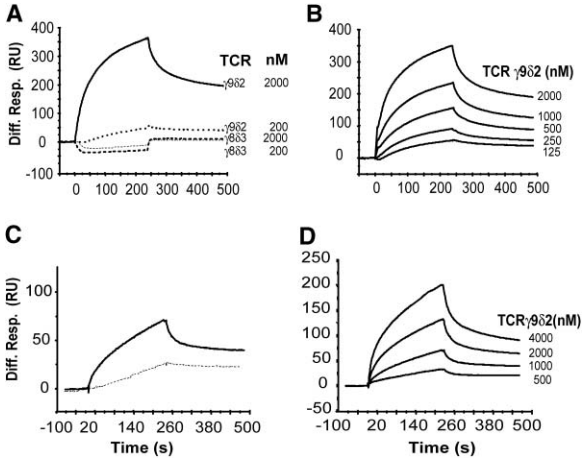

Figure 6. Surface Plasmon Resonance Analysis of TCR Binding to Apo A-I and F1

Soluble proteins were exposed to the sensorchip surface for 240 s (association phase) followed by a 240 s flow running (dissociation phase). Immobilized proteins were on sensorchips flow cell 2. Sensorgrams are representative of specific interactions (differential response) where nonspecific binding that occurred on flow cell 1 (with no protein immobilized) was deduced from binding that occurred on flow cell 2. Results are expressed as resonance units (RU) as a function of time in seconds.

(A and B) Overlay sensorgrams for SPR analysis of soluble TCRγδ protein binding to immobilized apo A-I. Amount of immobilized apo A-I protein was 2260 RU on flow cell 2. Comparative sensorgrams of soluble Vγ9Vδ2TCR and Vγ8Vδ3TCR (200 and 2000 nM) binding onto immobilized apo A-I (A). Vγ9Vδ2TCR was injected at concentrations ranging from 125 nM to 2 μM (B). The apparent kinetic constants of the interaction were $k_a = 8.8e^3 \pm 6.08\ e^2\ M^{-1}s^{-1}$, $k_d = 7.13\ e^{-3} \pm 6.76\ e^{-5}\ s^{-1}$, and $K_D = 8.1\ e^{-7}$ M.

(C and D) Comparative SPR sensorgrams of soluble TCR binding to immobilized F1. Amount of immobilized F1 was 22440 RU on flow cell 2. Comparative sensorgrams of purified monomeric G115 TCR (Vγ9Vδ2, full line) and 73R9 TCR (Vγ8Vδ3, dotted line) binding to immobilized F1 (C). Proteins were injected at a concentration of 2 μM. Monomeric G115 soluble TCR (Vγ9Vδ2) was injected at concentrations ranging from 0.5 to 4 μM (D). The apparent kinetic constants of the interaction were $k_a = 1.68\ e^3 \pm 2.16\ M^{-1}s^{-1}$, $k_d = 2.54\ e^{-3} \pm 2.9\ e^{-5}\ s^{-1}$, and $K_D = 1.51\ e^{-6}$M.

contrast, washing antigen-presenting cells after pulsing with exogenous IPP abrogates Vγ9Vδ2 T cell activation. This work did not explore the possibility that secreted IPP could be stably associated to components of the cell surface or from the extracellular medium. The stable modification of apo A-I or F1 by endogenous phosphoantigens is currently under investigation and beyond the scope of the present work. Although blocking T cell activation with anti-apo A-I or anti-βF1 is (partially) reversed by exogenous addition of IPP, this reversal does not exclude a simultaneous recognition of F1 with endogenous phosphoantigenic ligands (Figure 4). However, this hypothesis may not fully explain the reactivity of Vγ9Vδ2 T cells to phosphoantigens. Indeed, IPP or other phosphoantigens can readily activate Vγ9Vδ2 T cells in the absence of any third party cells (Gober et al., 2003; Lang et al., 1995; Morita et al., 1995). The implication of F1 in IPP presentation would thus imply F1 expression by γδ T cells. Despite this, all our attempts to detect F1 on T cell effectors have failed so far (data not shown).

Recognition by Vγ9Vδ2 T cells of F1, which is expressed on healthy tissues such as hepatocytes and endothelial cells, raises issues about the mechanisms preventing overt activation of Vγ9Vδ2 T cells by these self-structures under physiological conditions. As hypothesized above, F1 may not be recognized per se in vivo but in association with phosphorylated ligands derived from microbial agents or stressed/transformed cells. Moreover, inhibitory NK receptors, which are frequently found on Vγ9Vδ2 T cells and have been shown to raise their activation threshold (Fisch et al., 1997; Halary et al., 1997), may prevent recognition of F1-bearing normal cells that express high levels of NK receptor ligands, such as HLA class I molecules.

In conclusion, this study demonstrates cognate interactions between a human γδ TCR and several defined structures, namely F1-ATPase and apo A-I. Together with the high expression of LDL receptors and apoE in intraepithelial γδ lymphocytes (Fahrer et al., 2001), and studies documenting apoE binding to F1 (Mahley et al., 1989), these data open a new field of investigations linking lipid metabolism and antitumor immunosurveillance.

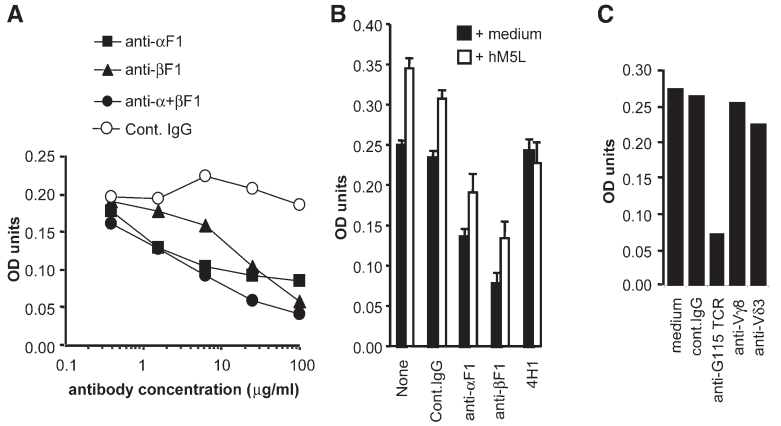

Figure 7. Soluble TCR Binding to Purified F1

ELISA plate microwells were coated with purified bovine ATP synthase (F1-F0) and saturated with BSA. Soluble biotinylated monomeric G115 TCR (Vγ9Vδ2) was then added, and TCR binding was measured by a standard streptavidin-alkaline phosphatase colorimetric test.

(A) Antibodies against α and β subunits of F1 (7H10 and 3D5, respectively) were added as competitors at the indicated concentration prior to the addition of soluble TCR.

(B) Some wells (white bars) were incubated with an apo A-I preparation (hM5L, 50 μg/ml) and washed prior to the addition of antibodies and soluble TCR. All antibodies were added at the concentration of 100 μg/ml.

(C) To assess TCR binding specificity, G115 TCR was mixed with the indicated TCR antibodies prior to addition of the mixture to F1-F0-coated wells.

Experimental Procedures

Tumor Cell Lines, T Cell Clones, and Cultures

Daudi, Raji, RPMI 8226, K562, Jurkat, Molt-4, U937, SK-NEP, G401, G402, and 786.0 were obtained from ATCC. Awells (EBV$^+$ lymphoblastoid B cell line) is from the International Histocompatibility Workshop (IHW#9090). C1R (HLA-A$^-$B$^-$-LCL) was obtained from Dr P. Lebouteiller (Toulouse, France). All tumor cell lines were cultivated in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) (Invitrogen) except for serum deprivation experiments. In this case, cells were washed once in RPMI 1640, incubated for 1 hr at 37°C in serum-free culture medium (hybridoma SFM, Invitrogen), pelleted, and reincubated for at least 16 hr at 37°C in SFM before use. The G25 (Vγ9Vδ2) and 73R9 (Vγ8Vδ3) clones were obtained as described for G42 and G115 (Allison et al., 2001; Davodeau et al., 1993) by anti-Vδ monoclonal antibody selection and subsequent amplification with PHA and IL2 and cloning.

Fluorescence Analysis and Antibodies

Surface immunofluorescence stainings were performed in PBS containing 1% BSA and devoid of serum using FITC-conjugated goat F(ab)$'_2$ anti-mouse Ig antibody (Caltag) as the second step reagent. Irrelevant isotype-matched control antibodies were used as negative controls. In apolipoprotein binding experiments, serum-deprived cells were incubated with serum or purified protein preparations in PBS containing 1% BSA at room temperature 30 min prior to antibody staining. 7H10 (anti-α-ATP synthase) and 3D5 (anti-β-ATP synthase) are from Molecular Probes. 4H1 (anti-human apo A-I) (Collet et al., 1997) was obtained from Dr Y. Marcel (Ottawa, Canada). TNFα accumulation in stimulated fresh PBL was analyzed after activation with indicated latex beads for 4 hr in the presence brefeldin A. Cells were then stained with antibodies to lymphocyte subsets, fixed, and permeabilized, and TNFα accumulation was analyzed after intracellular staining by flow cytometry. PMA/ionomycin stimulation was used as a control for TNFα-producing cells in the total population. Anti-Vδ2 (IMMU389), anti-NK (IM1847, anti CD158a), and anti-$\alpha\beta$TCR (BMA031) used for lymphocyte subset stainings are from Beckman Coulter.

Generation of M5A12D10 Hybridoma and Antibody Purification

Balb/c mice were injected intraperitoneally four times at 2 week intervals with 15×10^6 Daudi cells washed and resuspended in PBS. Hybridoma were obtained by fusion of spleen cells with P3X63Ag8 myeloma cells and were selected on the basis of tumor cell staining. Subcloned hybridoma were subsequently amplified in SFM, and antibodies were purified on protein G affinity columns (Pharmacia), neutralized, dialyzed against PBS, and concentrated (Harlow and Lane, 1988). Finally, antibodies were tested for their ability to modulate lysis of Daudi cells by $\gamma\delta$ effectors, leading to the selection of the M5A12D10 antibody (IgG1).

Purified Proteins

The human and bovine ligands of M5A12D10 (hM5L and bM5L, respectively) were isolated by affinity chromatography: human and fetal calf serum diluted 1/20 in 3 M NaCl and 50 mM Tris (pH 7) were passed through the column carrying the covalently attached antibody. After washing (last wash was in 3 M NaCl, 10 mM Tris [pH 7]), bound proteins were eluted with 100 mM glycine (pH 2.7). Isolation of apo A-I from high-density lipoproteins (HDL apo A-I) by ion-exchange chromatography has been already described (Mezdour et al., 1987). Purity of apo A-I was checked by Western blot analyses by using different antibodies directed against human apoB, apo A-II, apo-C, and apo A-I. The apo A-I homogeneity was more than 99% (as measured by densitometry after SDS-PAGE and silver staining). A purified preparation of apo A-I was obtained from Sigma Co. Purified bovine heart mitochondrial ATP synthase (F1 and F1/FO complexes) (Lutter et al., 1993) was obtained from John E. Walker (Cambridge, UK).

Production of Soluble Recombinant TCR

The G115 (Vγ9Vδ2) (Allison et al., 2001) and 73R9 (Vγ8Vδ3) extracellular γ and δ chains (the latter carrying a short 3′ biotin tag) were expressed in *Escherichia coli* then refolded together by rapid dilution in 1 liter of 1 M L-arginine, 0.1 M Tris-HCl, (pH 8.0), and 0.2 mM reduced/0.2 mM oxidized glutathione. After dialysis against 10 mM Tris-HCl (pH 8.0), concentration by cation exchange chromatography at (pH 5.5), and purification by size exclusion chromatography at (pH 8.0), the refolded protein was biotinylated for 4 hr at 30°C with 6 μg/ml BirA, and excess of free biotin was removed by dialysis against 10 mM Tris-HCl (pH 8.0), 150 mM NaCl.

Protein Identification by Proteomic Analysis and Mass Spectrometry

Immunopurified material was analyzed by using one-dimensional electrophoresis and visualized by Coomassie blue staining. Protein bands were excised from the gel and subjected to several washing steps, reduction alkylation reaction, in-gel trypsin digestion with modified trypsine (Promega, Madison, WI) at 25 ng/μl in 50 mM NH_4HCO_3, and finally followed by peptide extraction. The peptides purified with ZipTip C18 (Millipore) were mixed with equal volumes (0.5 ml) of a saturated a-cyano-4-hydroxycinnamic acid in 50% acetonitrile, 0.1% TFA onto the MALDI target and allowed to air dry. Peptide mass fingerprinting was obtained by using a PE Biosystems MALDI-TOF mass spectrometer (Voyager DE STR, Foster City, CA, USA) on each protein band. Unknown proteins were identified with the database fitting program MS-Fit (Protein Prospector, http://prospector.ucsf.edu) and searching against all eukaryotic entries in Swiss Prot and NCBI nonredundant protein databases. We considered the identification positive when a minimum of four measured peptide masses were matched and provided at least around 20% sequence coverage. Mass accuracy of 10 ppm was obtained with internal calibration by using autodigestion peaks of trypsin (M+H+, 842.51, 2211.10, and 2283.18).

Chromium and Cytokine Release Assays

2h-^{51}Cr-release assays were performed in standard conditions except for the use of serum-free conditions in some experiments: in sensitization experiments with apolipoprotein preparations, target tumor cells were serum deprived as described for FACS analysis, loaded with ^{51}Cr (100 μCi/10^6 cells, 1 hr, 37°C), extensively washed in RPMI, and resuspended in SFM medium. Effector T cells cultivated in serum-containing medium were washed extensively in RPMI, incubated for 2 hr in SFM at 37°C, and resuspended in SFM medium. Target cells (3000/well, in triplicates) were first incubated with apolipoprotein preparations or serum-containing medium for 30 min at room temperature in 96-well round-bottom microculture plates prior to the addition of effector cells. Cells were then pelleted and incubated at 37°C for 2 hr. Supernatants were recovered for ^{51}Cr-release measurement. Spontaneous release (in the absence of effectors) was subtracted from experimental data and was in the 10%–30% range of maximum release (effector cells replaced by same volume of 0.1 M HCl). Specific lysis was calculated as the percentage of maximum release. For antibody blocking experiments, the percentage of inhibition was calculated according to the formula: percentage of inhibition = [(percentage of specific lysis without antibody)−(percentage of specific lysis with antibody)]/[percentage of lysis without antibody] × 100.

For cytokine release measurements, similar experiments were performed. Supernatants were harvested after 4 hr (TNFα) or 20 hr (IFNγ), and IFNγ was titrated by a specific ELISA technique, whereas TNFα concentration was assessed by a biological assay based on WEHI cells viability.

ELISA Assay for Soluble TCR Binding

Purified mitochondrial ATP synthase (F1-F0, 10 μg/ml) was coated on ELISA plates in 50 μl of 0.1 M sodium carbonate buffer (pH 9.6), overnight at 4°C. Wells were then saturated with BSA (10 mg/ml, 2 hr, 22°C) and washed (PBS, 0.1% tween 20). Soluble biotinylated G115 TCR (Vγ9Vδ2) was added in 60 μL (1 μg/ml final concentration) for 30 min at 22°C and wells were washed. Bound TCR was revealed by adding alkaline phosphatase-coupled streptavidin (Jackson Immuno-Research, 0.2 μg/ml, 30 min, 22°C) in 50 μL of PBS, 0.1% tween 20. After washing, N nitrophenyl dissodium was added (1 μg/ml) in 100 mL of alkaline phosphatase substrate buffer (8.7% diethanolamine, 0.02% NaN_3, and 0.1 g/L $MgCl_2$). In competition

experiments, anti-F1 and anti-apo A-I antibodies were added at the indicated concentration in 50μL 15 min prior to the addition of soluble TCR and were left in the medium. In order to study the effect of apo A-I on TCR binding to F1, hM5L (100 μg/ml) was added on F1-F0-coated, BSA-saturated wells for 1 hr in 0.1 M carbonate buffer (pH 8.3) at 22°C and wells were then washed. To study the specificity of TCR binding to immobilized F1-F0, anti-TCR antibodies were mixed with soluble TCR, and the mixture was added after 30 min to F1-coated, BSA-saturated wells. Optical density was measured at 405 nm with a 595 nm reference. Results are expressed as the mean optical density of triplicate wells after blank subtraction (wells without F1). Plate reading was performed before an optical density of 0.1 OD_{405} unit was reached in blank wells.

Immobilization of Proteins on Latex Beads

10^7 sulfate latex beads (Interfacial Dynamics Corp., Portland, OR) were washed in PBS and incubated with apolipoproteins (100 μg/ml), F1-ATPase (0.4 mg/ml), or a mixture of both under constant agitation at room temperature for 16 hr. Beads were then washed, saturated for 3 hr in PBS containing 1% BSA, and washed extensively in PBS before use. Control "empty" beads were similarly saturated with BSA. In stimulation experiments, beads were mixed with cells at a 1:1 ratio.

SPR (Biacore) Analysis

Apo A-I and F1 were immobilized by amine linkage on CM5 chips (Biacore AB) after NHS-EDC activation. Binding was analyzed in a Biacore 3000 apparatus. Soluble ligands were injected at a flow rate of 20 μl/min, exposed to the surface for 240 s (association phase), followed by a 240 s flow running during which the dissociation occurred. Between injections, in order to recover the prebinding baseline, the sensorchip surface was regenerated by a 5 μL injection of 0.01% SDS (15 s of contact time).

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References

Allison, T.J., Winter, C.C., Fournie, J.J., Bonneville, M., and Garboczi, D.N. (2001). Structure of a human gammadelta T-cell antigen receptor. Nature *411*, 820–824.

Bae, T.J., Kim, M.S., Kim, J.W., Kim, B.W., Choo, H.J., Lee, J.W., Kim, K.B., Lee, C.S., Kim, J.H., Chang, S.Y., et al. (2004). Lipid raft proteome reveals ATP synthase complex in the cell surface. Proteomics *4*, 3536–3548.

Beckman, E.M., Porcelli, S.A., Morita, C.T., Behar, S.M., Furlong, S.T., and Brenner, M.B. (1994). Recognition of a lipid antigen by CD1-restricted alpha beta+ T cells. Nature *372*, 691–694.

Brossay, L., Naidenko, O., Burdin, N., Matsuda, J., Sakai, T., and Kronenberg, M. (1998). Structural requirements for galactosylceramide recognition by CD1-restricted NK T cells. J. Immunol. *161*, 5124–5128.

Bukowski, J.F., Morita, C.T., and Brenner, M.B. (1994). Recognition and destruction of virus-infected cells by human gamma delta CTL. J. Immunol. *153*, 5133–5140.

Bukowski, J.F., Morita, C.T., Tanaka, Y., Bloom, B.R., Brenner, M.B., and Band, H. (1995). V gamma 2V delta 2 TCR-dependent recognition of non-peptide antigens and Daudi cells analyzed by TCR gene transfer. J. Immunol. *154*, 998–1006.

Bukowski, J.F., Morita, C.T., and Brenner, M.B. (1999). Human gamma delta T cells recognize alkylamines derived from microbes, edible plants, and tea: implications for innate immunity. Immunity *11*, 57–65.

Chambenoit, O., Hamon, Y., Marguet, D., Rigneault, H., Rosseneu, M., and Chimini, G. (2001). Specific docking of apolipoprotein A-I at the cell surface requires a functional ABCA1 transporter. J. Biol. Chem. *276*, 9955–9960.

Collet, X., Marcel, Y.L., Tremblay, N., Lazure, C., Milne, R.W., Perret, B., and Weech, P.K. (1997). Evolution of mammalian apolipoprotein A-I and conservation of antigenicity: correlation with primary and secondary structure. J. Lipid Res. *38*, 634–644.

Constant, P., Davodeau, F., Peyrat, M.A., Poquet, Y., Puzo, G., Bonneville, M., and Fournie, J.J. (1994). Stimulation of human gamma delta T cells by nonpeptidic mycobacterial ligands. Science *264*, 267–270.

Crowley, M.P., Fahrer, A.M., Baumgarth, N., Hampl, J., Gutgemann, I., Teyton, L., and Chien, Y. (2000). A population of murine gammadelta T cells that recognize an inducible MHC class Ib molecule. Science *287*, 314–316.

Das, B., Mondragon, M.O., Sadeghian, M., Hatcher, V.B., and Norin, A.J. (1994). A novel ligand in lymphocyte-mediated cytotoxicity: expression of the beta subunit of H+ transporting ATP synthase on the surface of tumor cell lines. J. Exp. Med. *180*, 273–281.

Davis, M.M., Boniface, J.J., Reich, Z., Lyons, D., Hampl, J., Arden, B., and Chien, Y. (1998). Ligand recognition by alpha beta T cell receptors. Annu. Rev. Immunol. *16*, 523–544.

Davodeau, F., Peyrat, M.A., Houde, I., Hallet, M.M., De Libero, G., Vie, H., and Bonneville, M. (1993). Surface expression of two distinct functional antigen receptors on human gamma delta T cells. Science *260*, 1800–1802.

De Libero, G., Casorati, G., Giachino, C., Carbonara, C., Migone, N., Matzinger, P., and Lanzavecchia, A. (1991). Selection by two powerful antigens may account for the presence of the major population of human peripheral gamma/delta T cells. J. Exp. Med. *173*, 1311–1322.

Fahrer, A.M., Konigshofer, Y., Kerr, E.M., Ghandour, G., Mack, D.H., Davis, M.M., and Chien, Y.H. (2001). Attributes of gammadelta intraepithelial lymphocytes as suggested by their transcriptional profile. Proc. Natl. Acad. Sci. USA *98*, 10261–10266.

Fisch, P., Malkovsky, M., Kovats, S., Sturm, E., Braakman, E., Klein, B.S., Voss, S.D., Morrissey, L.W., De Mars, R., Welch, W.J., et al. (1990). Recognition by human V gamma 9/V delta 2 T cells of a GroEL homolog on Daudi Burkitt's lymphoma cells. Science *250*, 1269–1273.

Fisch, P., Meuer, E., Pende, D., Rothenfusser, S., Viale, O., Kock, S., Ferrone, S., Fradelizi, D., Klein, G., Moretta, L., et al. (1997). Control of B cell lymphoma recognition via natural killer inhibitory receptors implies a role for human Vgamma9/Vdelta2 T cells in tumor immunity. Eur. J. Immunol. *27*, 3368–3379.

Girardi, M., Oppenheim, D.E., Steele, C.R., Lewis, J.M., Glusac, E., Filler, R., Hobby, P., Sutton, B., Tigelaar, R.E., and Hayday, A.C. (2001). Regulation of cutaneous malignancy by gammadelta T cells. Science *294*, 605–609.

Girardi, M., Glusac, E., Filler, R.B., Roberts, S.J., Propperova, I., Lewis, J., Tigelaar, R.E., and Hayday, A.C. (2003). The distinct contributions of murine T cell receptor (TCR)gammadelta+ and TCRalphabeta+ T cells to different stages of chemically induced skin cancer. J. Exp. Med. *198*, 747–755.

Gober, H.J., Kistowska, M., Angman, L., Jeno, P., Mori, L., and De Libero, G. (2003). Human T cell receptor gammadelta cells recognize endogenous mevalonate metabolites in tumor cells. J. Exp. Med. *197*, 163–168.

Groh, V., Steinle, A., Bauer, S., and Spies, T. (1998). Recognition of stress-induced MHC molecules by intestinal epithelial gammadelta T cells. Science *279*, 1737–1740.

Halary, F., Peyrat, M.A., Champagne, E., Lopez-Botet, M., Moretta,

A., Moretta, L., Vie, H., Fournie, J.J., and Bonneville, M. (1997). Control of self-reactive cytotoxic T lymphocytes expressing gamma delta T cell receptors by natural killer inhibitory receptors. Eur. J. Immunol. *27*, 2812–2821.

Harlow, E., and Lane, D. (1988). Antibodies: A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press), pp. 312.

Lang, F., Peyrat, M.A., Constant, P., Davodeau, F., David-Ameline, J., Poquet, Y., Vie, H., Fournie, J.J., and Bonneville, M. (1995). Early activation of human V gamma 9V delta 2 T cell broad cytotoxicity and TNF production by nonpeptidic mycobacterial ligands. J. Immunol. *154*, 5986–5994.

Lutter, R., Saraste, M., van Walraven, H.S., Runswick, M.J., Finel, M., Deatherage, J.F., and Walker, J.E. (1993). F1F0-ATP synthase from bovine heart mitochondria: development of the purification of a monodisperse oligomycin-sensitive ATPase. Biochem. J. *295*, 799–806.

Mahley, R.W., Hui, D.Y., Innerarity, T.L., and Beisiegel, U. (1989). Chylomicron remnant metabolism. Role of hepatic lipoprotein receptors in mediating uptake. Arteriosclerosis *9*, I14–I18.

Malkovska, V., Cigel, F., and Storer, B.E. (1994). Human T cells in hu-PBL-SCID mice proliferate in response to Daudi lymphoma and confer anti-tumour immunity. Clin. Exp. Immunol. *96*, 158–165.

Martinez, L.O., Jacquet, S., Esteve, J.P., Rolland, C., Cabezon, E., Champagne, E., Pineau, T., Georgeaud, V., Walker, J.E., Terce, F., et al. (2003). Ectopic beta-chain of ATP synthase is an apolipoprotein A-I receptor in hepatic HDL endocytosis. Nature *421*, 75–79.

Mezdour, H., Clavey, V., Kora, I., Koffigan, M., Barkia, A., and Fruchart, J.C. (1987). Anion-exchange fast protein liquid chromatographic characterization and purification of apolipoproteins A-I, A-II, C–I, C–II, C–III0, C–III1, C–III2 and E from human plasma. J. Chromatogr. *414*, 35–45.

Moody, D.B., Reinhold, B.B., Guy, M.R., Beckman, E.M., Frederique, D.E., Furlong, S.T., Ye, S., Reinhold, V.N., Sieling, P.A., Modlin, R.L., et al. (1997). Structural requirements For glycolipid antigen recognition by Cd1b-restricted T cells. Science *278*, 283–286.

Morita, C.T., Beckman, E.M., Bukowski, J.F., Tanaka, Y., Band, H., Bloom, B.R., Golan, D.E., and Brenner, M.B. (1995). Direct presentation of nonpeptide prenyl pyrophosphate antigens to human gamma delta T cells. Immunity *3*, 495–507.

Moser, T.L., Stack, M.S., Asplin, I., Enghild, J.J., Hojrup, P., Everitt, L., Hubchak, S., Schnaper, H.W., and Pizzo, S.V. (1999). Angiostatin binds ATP synthase on the surface of human endothelial cells. Proc. Natl. Acad. Sci. USA *96*, 2811–2816.

Murao, K., Terpstra, V., Green, S.R., Kondratenko, N., Steinberg, D., and Quehenberger, O. (1997). Characterization of CLA-1, a human homologue of rodent scavenger receptor BI, as a receptor for high density lipoprotein and apoptotic thymocytes. J. Biol. Chem. *272*, 17551–17557.

Ramsburg, E., Tigelaar, R., Craft, J., and Hayday, A. (2003). Age-dependent requirement for gammadelta T cells in the primary but not secondary protective immune response against an intestinal parasite. J. Exp. Med. *198*, 1403–1414.

Spada, F.M., Grant, E.P., Peters, P.J., Sugita, M., Melian, A., Leslie, D.S., Lee, H.K., van Donselaar, E., Hanson, D.A., Krensky, A.M., et al. (2000). Self-recognition of CD1 by gamma/delta T cells: implications for innate immunity. J. Exp. Med. *191*, 937–948.

Stanic, A.K., Shashidharamurthy, R., Bezbradica, J.S., Matsuki, N., Yoshimura, Y., Miyake, S., Choi, E.Y., Schell, T.D., Van Kaer, L., Tevethia, S.S., et al. (2003). Another view of T cell antigen recognition: cooperative engagement of glycolipid antigens by Va14Ja18 natural TCR. J. Immunol. *171*, 4539–4551.

Tanaka, Y., Sano, S., Nieves, E., De Libero, G., Rosa, D., Modlin, R.L., Brenner, M.B., Bloom, B.R., and Morita, C.T. (1994). Nonpeptide ligands for human gamma delta T cells. Proc. Natl. Acad. Sci. USA *91*, 8175–8179.

Wu, J., Groh, V., and Spies, T. (2002). T cell antigen receptor engagement and specificity in the recognition of stress-inducible MHC class I-related chains by human epithelial gamma delta T cells. J. Immunol. *169*, 1236–1240.